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**14. ABSTRACT**
The greatest factor for the development of prostate adenocarcinoma is advanced age. Emerging evidence suggests that molecular alterations in the aged prostate microenvironment mediated by stromal aging and senescence are key factors regulating carcinogenesis and neoplastic progression. We used normal mouse prostate epithelial and adjacent stromal cells from young and old animals, microdissected *in situ*, to identify factors altered by the aged stroma that may place the prostate gland at risk for developing prostate cancer. Expression profiling demonstrated clear differences in gene expression between old and young prostate stroma, with 219 genes exhibiting significant transcript abundance levels ($p<0.005$). Transcripts for Type I and III collagen were among the genes most substantially altered with aging. qRT-PCR confirmed the lower expression of Col1a2 and Col3a1 in the aged prostate stroma and immunofluorescence detection for Type-I collagen revealed a disorganized collagen matrix in the aged prostate. The alterations in the collagen network affect the structural and signaling properties of the extracellular matrix and in turn could plausibly facilitate carcinogenesis and neoplastic progression. The aged-expression profiling also demonstrated the up-regulation of several chemokines (Ccl8, Ccl7), and factors that respond to pro-inflammatory agonist and senescent inducers, such as ApoD in the aged stroma. In summary, the transcriptional profile of the aged prostate microenvironment provides novel data regarding senescence-associated candidate genes important in prostate carcinogenesis. Further studies are necessary in order to provide a functional significance of these alterations with the ultimate goal of supplying strong preclinical data regarding age-associated prostatic stromal factors influencing carcinogenesis that can be translated into novel human studies of prostate cancer prevention.

**15. SUBJECT TERMS**

Microarray, aging, microenvironment, prostate

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## Table of Contents

**Page**

**INTRODUCTION:**

The greatest single risk factor for the development of prostate adenocarcinoma is advanced age(1-5). Emerging evidence suggests that molecular alterations in the aged prostate microenvironment mediated by stromal aging and senescence are key factors regulating carcinogenesis and neoplastic progression(6, 7). However, no functional studies have been reported that definitively provide mechanistic evidence of cause and effect. This proposal is designed to investigate the role of the aged-stroma microenvironment in prostate carcinogenesis. Our *hypothesis is that gene expression differences can be identified between normal stroma from young vs. old mice, and that candidate genes identified in the aged-stroma have the potential to influence the proliferation, survival, or invasive capabilities of adjacent transformed epithelium via paracrine mechanisms.* The ultimate goal of this proposal is to provide strong preclinical data that can be translated into novel human studies of prostate cancer prevention.

**BODY:**

We performed gene expression profiling using RNA isolated from micro-dissected benign mouse prostate stroma from young (n=5; aged 4 months) and old (n=5; aged 24 months) wild-type C57BL/6 mice. RNA was amplified and hybridized to a mouse prostate-specific cDNA microarray (MPEDB arrays) (Figure 1). A mouse gold standard RNA was used as a reference for all microarrays.

*Laser Captured Microdissected Stroma-Sample purity:* The prostate gland is composed primarily of secretory luminal epithelium, basal epithelium, and stromal cells. The prostatic stroma compartment comprises different cells types: a) the stroma adjacent to the prostatic ducts is composed primarily of smooth muscle cells and fibroblasts and b) endothelial and inflammatory cells present between the gland ducts. Since the focus of this study was to identify age related stromal and epithelial factors we laser captured microdissected the adjacent stroma surrounding the epithelial cells and the luminal epithelial cells.

The Cell-type specific purity was verified by analyzing the expression level (log2 ratio) of known stromal and epithelial markers obtained in the microarray experiment (Figure 2a). To further characterize the relationships between the epithelial and stroma samples and between age groups, we performed Principal Component Analysis (PCA) for all the genes in the arrays including all the stroma and epithelial samples from young and old groups (Figure 2b). PCA clearly identified a subset of genes that discriminated between epithelial and stroma samples, suggesting that the major differences between samples (53% of the total variance) results from the differential expression of large numbers of genes between the stroma and epithelial compartments. In summary, we were able to verify that the stroma samples were enriched in stroma cells compared to the epithelial samples and vice-versa, and that the differential expressed genes between age groups within and between cell compartments is only a minor subset of genes representing a small percentage of the total variance among all the samples tested.

*Aged-expression profile in the mouse prostate microenvironment:* Expression profiling demonstrated clear differences in gene expression between old and young prostate stroma, with

39 genes exhibiting significant alterations ($p < 0.005$; Figure 3a). In order to expand the list of significant genes altered with aging, we performed an additional microarray experiment from laser captured microdissected stroma from a new set of young (n=12; 4 month-old) and old (n=12; 24 month-old) mice and used a microarray platform containing ~ 40.000 genes (Agilent customized array) compared to ~9000 genes present in the original analysis. We identified that 180 genes exhibited significant age-related changes ($p<0.005$; Figure 4). Statistical analysis for both data sets was carried out using Student's t-test (unpaired, two-tailed, unequal variance), and transcripts with P-values <0.005 were considered significantly altered between young and old stroma. Within the set of genes that overlapped between both arrays, 24 and 23 transcripts with P-values <0.005 were significantly altered in the MPEDB and Agilent arrays respectively. A Pearson correlation analysis considering the most significant altered genes from MPEDB and Agilent data set shows a correlation of 0.6 and 0.5 respectively. Among the genes showing a similar trend in both data sets are: Ccl8, Ccl7, ApoD, Sparc, Col1a2 Col3a1 and Lamb1, among others (Figure 5). To this end, we are in the process of identifying other candidate genes from the Agilent arrays.

Alteration of genes encoding extracellular matrix component in the aged prostate:

In our original analysis using the MPEDB array, transcripts encoding extracellular matrix components for Type I and Type III collagen (Col1a2, down 4-fold and Col3a1 down 3-fold, respectively) were among the genes most substantially altered with aging. Quantitative RT-PCR confirmed the lower expression of Col1a2 and Col3a1 in the aged prostate stroma (Figure 6a). Interestingly, although immunofluorescence detection for Type-I and Type-III collagen did not show substantial differences at the protein level between young and old prostate tissue, confocal microscopy revealed a disorganized collagen matrix in the aged prostate microenvironment when compared to young prostate (Figure 6b). Previous studies have demonstrated the effect of fibrillar collagen and substrate rigidity on epithelial cell proliferation, apoptosis, and motility in culture systems, suggesting a direct role of the collagen network on the behavior of epithelial cells (8-10). Taken together, we hypothesized that the disorganized collagen matrix and the diminished expression of Col1a2 and Col3a1 in the aged prostate microenvironment may have an effect on the structural and signaling properties of the extracellular matrix. In turn, a dysfunctional ECM in the aged prostate could plausibly facilitate carcinogenesis and neoplastic progression.

Up-regulation of lipoprotein D: Apolipoprotein D (ApoD) is a member of the lipocalin superfamily of protein transporters that is implicated in the pathogenesis of neurodegenerative diseases and is regulated by androgens in both breast and prostate cells (11, 12). Based on the transcriptional profiles of the aged stroma from both the MPEDB and Agilent arrays, we identify ApoD as a transcript up-regulated in the aged microdissected stroma. Quantitative RT-PCR confirmed the higher expression of ApoD in the aged prostate stroma (up 4-fold; Figure 7a). To begin to address the relevance of factors identified in the aged murine prostate stroma with human senescence/aging transcriptional profiles, the transcript level of Human APOD was evaluated by RT-PCR in pre-senescent and senescent human fibroblast induced to senesce *in vitro* by different means ($H_2O_2$ and overexpression of p16). As shown in Figure 7b, APOD transcript is elevated in senescent cells compared to pre-senescent fibroblast. Our finding is supported by previous reports demonstrating an increase of ApoD transcript levels in the brain of

old mice and post-mortem human subjects (68±83-year-old) as well as in cultured cells as a response to inflammation and senescent inducers (13-17)

Studies of the Drosophila ApoD ortholog, GLaz, provide context for the potential influence of ApoD expression on cytoprotection and cell survival (18, 19). Overexpression of Glaz increased resistance to stresses that included starvation, hyperoxia and hypoxia, and resulted in the extension of organismal lifespan(18). Conversely, loss of GLaz resulted in the reduction of Drosophila stress resistance and lifespan, consistent with APOD being part of a defense system that is activated in the setting of oxidative stress, or incited by exogenous environmental factors or intrinsic events such as aging or neoplasia (19). Studies of prostate cancer have demonstrated elevated APOD protein levels in prostate intraepithelial neoplasia and prostate carcinoma (20), but associations between APOD expression and aging in the context of cancer have not been reported.

Intraprostatic inflammatory cells in aged mice: Recent studies have identified a role for inflammation in the development and progression of several cancers, such as liver, large intestine and stomach. However, a direct role of inflammation in prostate carcinogenesis and progression has yet to be identified (Reviewed in (20, 21)). During the microdissection of the mouse prostate stroma we noticed an increased number of inflammatory cells in the prostate from old animals. Immunohistochemistry analysis for CD45 (leukocytes) reveals an increase number of leukocytes in the prostates from old animals (Figure 8a). To further characterize the type of inflammatory cells present in the old prostate we used a CD3, B220 and F4/80 antibodies that recognize T lymphocytes, B lymphocytes and Macrophages respectively. As shown in Figure 8(b-d), the three cell types were present in the prostate. Consistent with our observation, studies in normal aged winstar rats demonstrated increase levels of lymphocytes in the prostate(22, 23).

Even though the laser capture microdissection of the adjacent stroma was performed very carefully trying to capture the fibroblast and smooth muscle cells adjacent to the prostatic epithelium, our transcriptional profile analysis revealed an increase in transcript levels of several genes expressed by inflammatory cells, such as Igh-6, Igj and Igk-V8 (Figure 3). IHC analysis demonstrated the presence of CD3, B220 and F4/80 positive cells within the adjacent stroma and epithelial compartment. Thus, it is not surprising to find transcripts encoded specifically by inflammatory cells in our transcriptional profile from the aged stroma. These results emphasized the importance of IHC and/or in situ hybridization analyses in order to identify the specific cells type expressing the candidate factor.

Studies in senescent cells and its secretory phenotype provide context for the potential influence of secreted growth factors, chemokines and cytokines in the infiltration of leukocytes and macrophages into the aged prostate (6, 24, 25). In our transcriptional profile analyses, several chemokines such as Ccl8, Ccl7 and Ccl5 and cytokines such as Gdf15, Cklf and Ebi3 were up-regulated in the LCM stroma from old animals (Figures 3 and 4). The up-regulation of Ccl8 was confirmed by RT-PCR; however to this end we have not been able to determine whether Ccl8 is being expressed in the fibroblast/smooth muscle cells, in the infiltrating cells or both. Nevertheless, preliminary experiments in our laboratory demonstrated that CCL8 is up-regulated at the transcript level in human prostatic senescent cells overexpressing p16. Interestingly, our transcriptional profile, not only identified chemoattractant factors, but also, factors that appear to respond to an inflammatory state, such as ApoD which has been demonstrated to be up-regulated in the presence of pro-inflammatory agonist.

Taken together, we are now interested in determining if the aged/senescent fibroblast/smooth muscle cells are secreting chemokines and cytokines that attract inflammatory cells to the prostate tissue or the inflammatory cells are present prior to an aged microenvironment phenotype.

**KEY RESEARCH ACCOMPLISHMENT:**

- We have identified age-dependent differences in gene expression from stroma cells isolated from its *in situ* environment from old and young mouse prostate tissues.
- We have validated the expression levels of several transcripts identified in this study such as Col1a2, Col3a1, Ccl8 and ApoD by RT-PCR.
- By immunofluorescent analysis for Collagen Type I, we were able to show the presence of a disorganized collagen matrix in the aged prostate microenvironment when compared to young prostate.
- We have identified ApoD as a factor altered both in *in vivo* aged stroma cells and *in vitro* human senescent cells.
- We demonstrated that high number of infiltrating cells is present in the prostate from aged mice. And it coincides with the up-regulation of genes involved in the inflammatory response.

**REPORTABLE OUTCOMES:**

Poster presentation:

*Poster title:* "Expression Profiles of the Aged Prostate Microenvironment Identify Alterations in Transcripts Encoding Extracellular Matrix Proteins: Implications for Prostate Carcinogenesis" Presented at the Translational Research at the Aging and Cancer Interface (AACR) February 20-23 2007.

Manuscripts in preparation:

1. Identification of Molecular Alterations in the mouse aged prostate microenvironment.

**CONCLUSION:**

Gene expression differences are evident between young and aged prostate stroma. The disorganized collagen matrix and the diminished expression of Col1a2 and Col3a1 in the aged prostate microenvironment may have an effect on the structural and signaling properties of the extracellular matrix. In turn, a dysfunctional ECM in the aged prostate could plausibly facilitate carcinogenesis and neoplastic progression. Further research to analyze the effect of young and old collagen in epithelial cell behavior is underway.

The prostate from old animals contains a higher number of infiltrating cells, including B lymphocytes, T lymphocytes and macrophages. The presence of these infiltrates might be a consequence of chemokine and cytokine factors secreted by the old prostate stroma, creating a microenvironment that favors neoplastic progression.

Additionally, the up-regulation of ApoD in the aged mouse prostate stroma and in human prostatic fibroblast induced to senesce *in vitro*, provide context for the potential influence of ApoD expression in the aged prostate on cytoprotection and cell survival. Further functional studies of the role of ApoD in cellular senescence and its down stream effect on prostate carcinogenesis are necessary and are underway.

It is anticipated that the identification of factors altered in the aged stroma and the study of the inflammatory cells present in the old prostate will prove invaluable for determining the underlying causes of the disruption of the homeostatic balance between the stroma and epithelium and what connection there is to prostate cancer, if any.

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**APPENDICES:**

Abstract for porter presented at the Translational Research at the Aging and Cancer Interface (AACR) February 20-23 2007

**Expression Profiles of the Aged Prostate Microenvironment Identify Alterations in Transcripts Encoding Extracellular Matrix Proteins: Implications for Prostate Carcinogenesis**

Daniella Bianchi-Frias and Peter S Nelson.
Division of Human Biology, Fred Hutchinson Cancer Research Center. Seattle, WA.

**Background:** The greatest single risk factor for the development of prostate adenocarcinoma is advanced age. Emerging evidence suggests that molecular alterations in the aged prostate microenvironment mediated by stromal aging and senescence are key factors regulating carcinogenesis and neoplastic progression. However, no functional studies have been reported that definitively provide mechanistic evidence of cause and effect. To investigate the role of the aged-stroma microenvironment in prostate carcinogenesis, we profiled the molecular changes in gene expression that occur with aging in the normal murine prostate stroma.

**Methods:** We performed gene expression profiling using RNA isolated from micro-dissected benign mouse prostate stroma from young (n=5; aged 4 months) and old (n=5; aged 24 months) animals. RNA was amplified and hybridized to a mouse prostate-specific cDNA microarray. Age-associated differential expression of candidate transcripts was confirmed by quantitative RT-PCR and immunohistochemitry.

**Results:** Expression profiling demonstrated clear differences in gene expression between old and young prostate stroma, with 39 genes exhibiting significant transcript abundance levels ($p<0.005$). Transcripts encoding extracellular matrix components for Type I and Type III collagen (Col1a2, down 4-fold and Col3a1 down 3-fold, respectively) were among the genes most substantially altered with aging. Quantitative RT-PCR confirmed the lower expression of Col1a2 and Col3a1 in the aged prostate stroma. Interestingly, although immunofluorescence detection for Type-I and Type-III collagen did not show substantial differences at the protein level between young and old prostate tissue, it revealed a disorganized collagen matrix in the aged prostate microenvironment when compared to young prostate.

**Conclusions:** Gene expression differences are evident between young and aged prostate stroma. The disorganized collagen matrix and the diminished expression of Col1a2 and Col3a1 in the aged prostate microenvironment may have an effect on the structural and signaling properties of the extracellular matrix. In turn, a dysfunctional ECM in the aged prostate could plausibly facilitate carcinogenesis and neoplastic progression.

# SUPPORTING DATA:

**Figure 1.** Experimental Design. A-B) Images of mouse prostate tissue sections (7uM) during the LCM procedure. A) Pre-Capture image shows stroma and epithelium (pink) with a focal area of inflammatory cells (ic) that will be avoided during capturing. B) stromal and epithelial cell region isolated on the LCM cap. C) RNA was isolated from LCM stroma and epithelium from young ( aged 4 months) and old (aged 24 months) wild-type C57BL/6 mice. LCM stroma and epithelial samples from 3 animals were pool from the dorsal and anterior prostatic lobes, for a total of 3 (4) pools per age group and cell type. RNA was amplified and hybridized to a mouse prostate-specific cDNA microarray (MPEDB arrays, 3 pools per age group) or a mouse customized Agilent array (4 pools per age group). A mouse gold standard RNA was used as a reference for all microarrays.

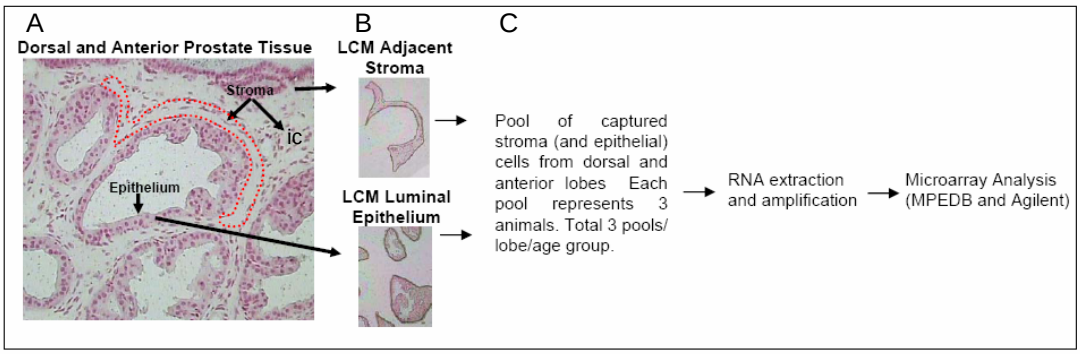


**Figure 2.** LCM cells purity. A) Transcript abundance levels (Log 2 ratios) obtained from MPEDB arrays of known stromal and epithelial markers in LCM samples acquired from mouse prostate stroma and epithelium. B) Principal Component Analysis for Dorsal Prostate Stroma and Epithelial samples from young and old animals. EO: old epithelium; EY: young epithelium; SO: old stroma; SY: young stroma

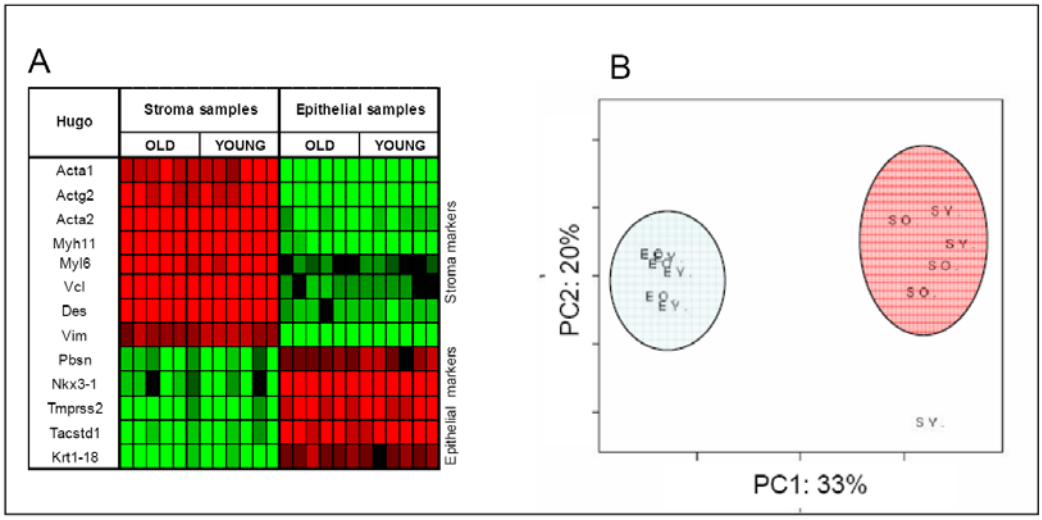
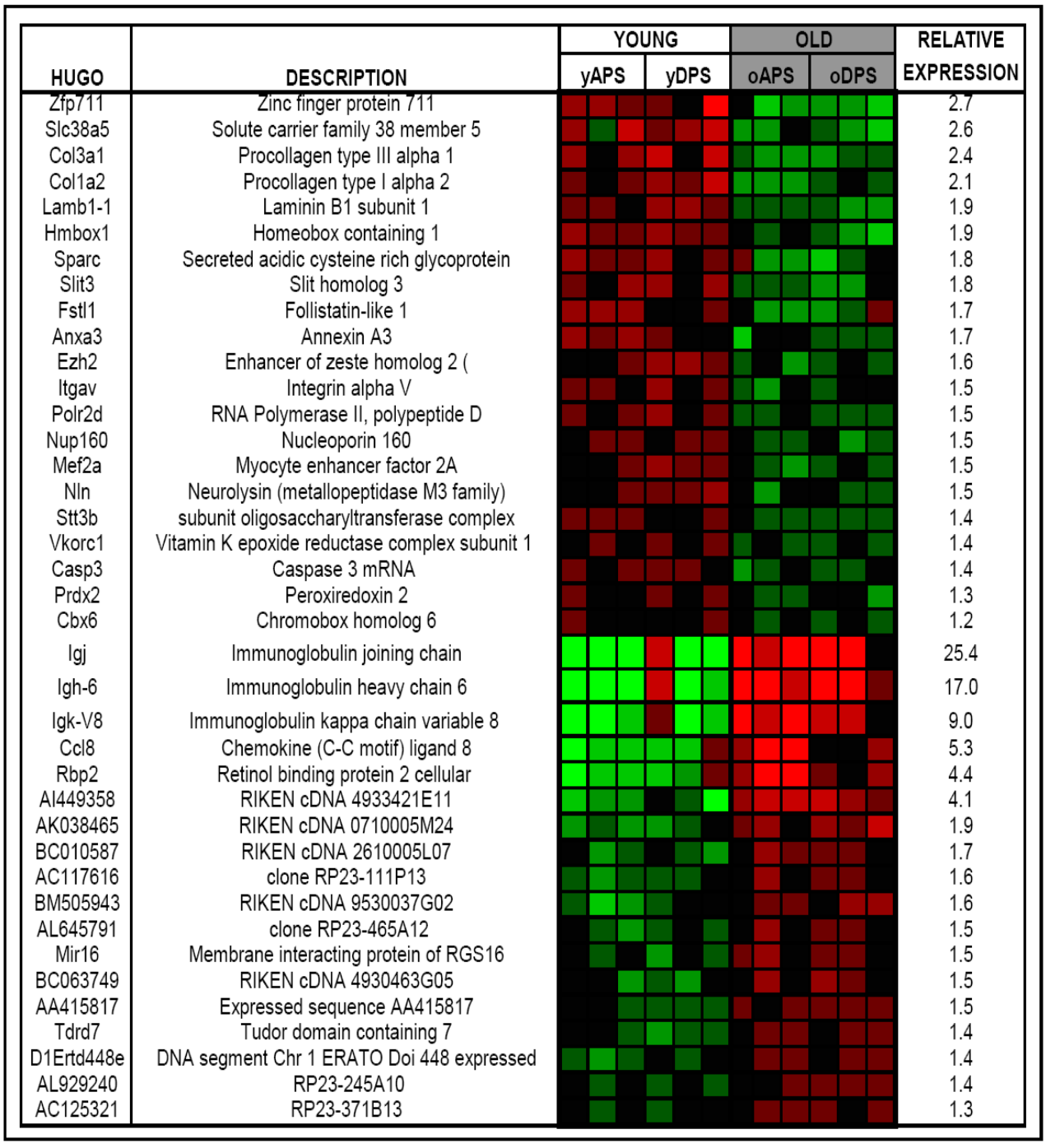


**Figure 3**. Differential gene expression between young and old prostate stroma obtained from MPEDB array. **Old (**24-months-old); **Young** (4-months-old). APS: Anterior and DPS: Dorsal Prostatic Stroma. **Green**: down-regulated and **Red**: up-regulated genes.



| HUGO | DESCRIPTION | YOUNG | | OLD | | RELATIVE EXPRESSION |
|---|---|---|---|---|---|---|
| | | yAPS | yDPS | oAPS | oDPS | |
| Zfp711 | Zinc finger protein 711 | | | | | 2.7 |
| Slc38a5 | Solute carrier family 38 member 5 | | | | | 2.6 |
| Col3a1 | Procollagen type III alpha 1 | | | | | 2.4 |
| Col1a2 | Procollagen type I alpha 2 | | | | | 2.1 |
| Lamb1-1 | Laminin B1 subunit 1 | | | | | 1.9 |
| Hmbox1 | Homeobox containing 1 | | | | | 1.9 |
| Sparc | Secreted acidic cysteine rich glycoprotein | | | | | 1.8 |
| Slit3 | Slit homolog 3 | | | | | 1.8 |
| Fstl1 | Follistatin-like 1 | | | | | 1.7 |
| Anxa3 | Annexin A3 | | | | | 1.7 |
| Ezh2 | Enhancer of zeste homolog 2 ( | | | | | 1.6 |
| Itgav | Integrin alpha V | | | | | 1.5 |
| Polr2d | RNA Polymerase II, polypeptide D | | | | | 1.5 |
| Nup160 | Nucleoporin 160 | | | | | 1.5 |
| Mef2a | Myocyte enhancer factor 2A | | | | | 1.5 |
| Nln | Neurolysin (metallopeptidase M3 family) | | | | | 1.5 |
| Stt3b | subunit oligosaccharyltransferase complex | | | | | 1.4 |
| Vkorc1 | Vitamin K epoxide reductase complex subunit 1 | | | | | 1.4 |
| Casp3 | Caspase 3 mRNA | | | | | 1.4 |
| Prdx2 | Peroxiredoxin 2 | | | | | 1.3 |
| Cbx6 | Chromobox homolog 6 | | | | | 1.2 |
| Igj | Immunoglobulin joining chain | | | | | 25.4 |
| Igh-6 | Immunoglobulin heavy chain 6 | | | | | 17.0 |
| Igk-V8 | Immunoglobulin kappa chain variable 8 | | | | | 9.0 |
| Ccl8 | Chemokine (C-C motif) ligand 8 | | | | | 5.3 |
| Rbp2 | Retinol binding protein 2 cellular | | | | | 4.4 |
| AI449358 | RIKEN cDNA 4933421E11 | | | | | 4.1 |
| AK038465 | RIKEN cDNA 0710005M24 | | | | | 1.9 |
| BC010587 | RIKEN cDNA 2610005L07 | | | | | 1.7 |
| AC117616 | clone RP23-111P13 | | | | | 1.6 |
| BM505943 | RIKEN cDNA 9530037G02 | | | | | 1.6 |
| AL645791 | clone RP23-465A12 | | | | | 1.5 |
| Mir16 | Membrane interacting protein of RGS16 | | | | | 1.5 |
| BC063749 | RIKEN cDNA 4930463G05 | | | | | 1.5 |
| AA415817 | Expressed sequence AA415817 | | | | | 1.5 |
| Tdrd7 | Tudor domain containing 7 | | | | | 1.4 |
| D1Ertd448e | DNA segment Chr 1 ERATO Doi 448 expressed | | | | | 1.4 |
| AL929240 | RP23-245A10 | | | | | 1.4 |
| AC125321 | RP23-371B13 | | | | | 1.3 |

**Figure 4.** Differential gene expression between young and old prostate stroma obtained from Agilent arrays. **Old (**24-months-old); **Young** (4-months-old). APS: Anterior and DPS: Dorsal Prostatic Stroma. **Green**: down-regulated and **Red**: up-regulated genes

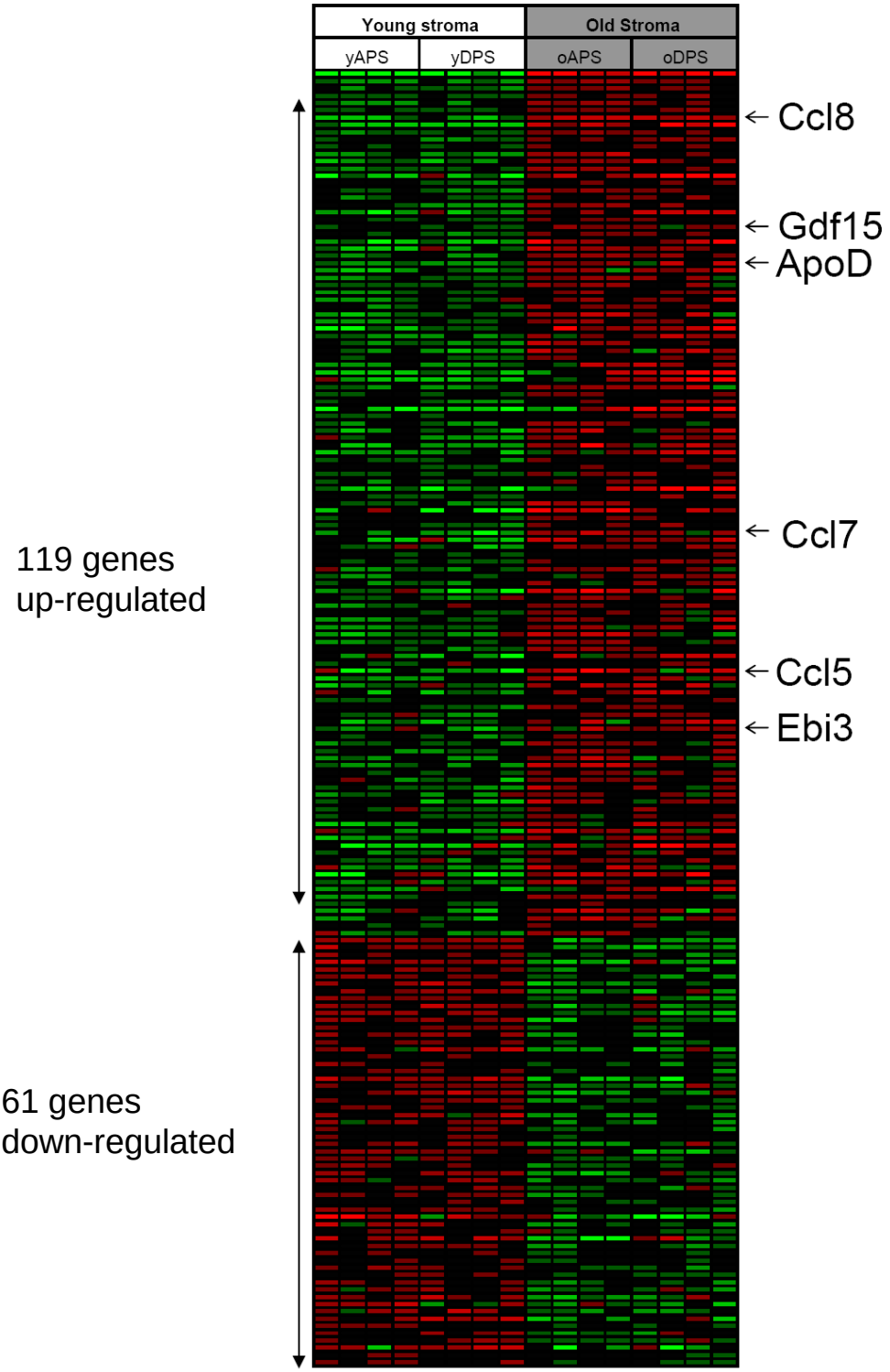


**Figure 5.** Correlation analysis between expression profiles obtained from MPEDB and Agilent data sets. Heatmaps has been median centered.

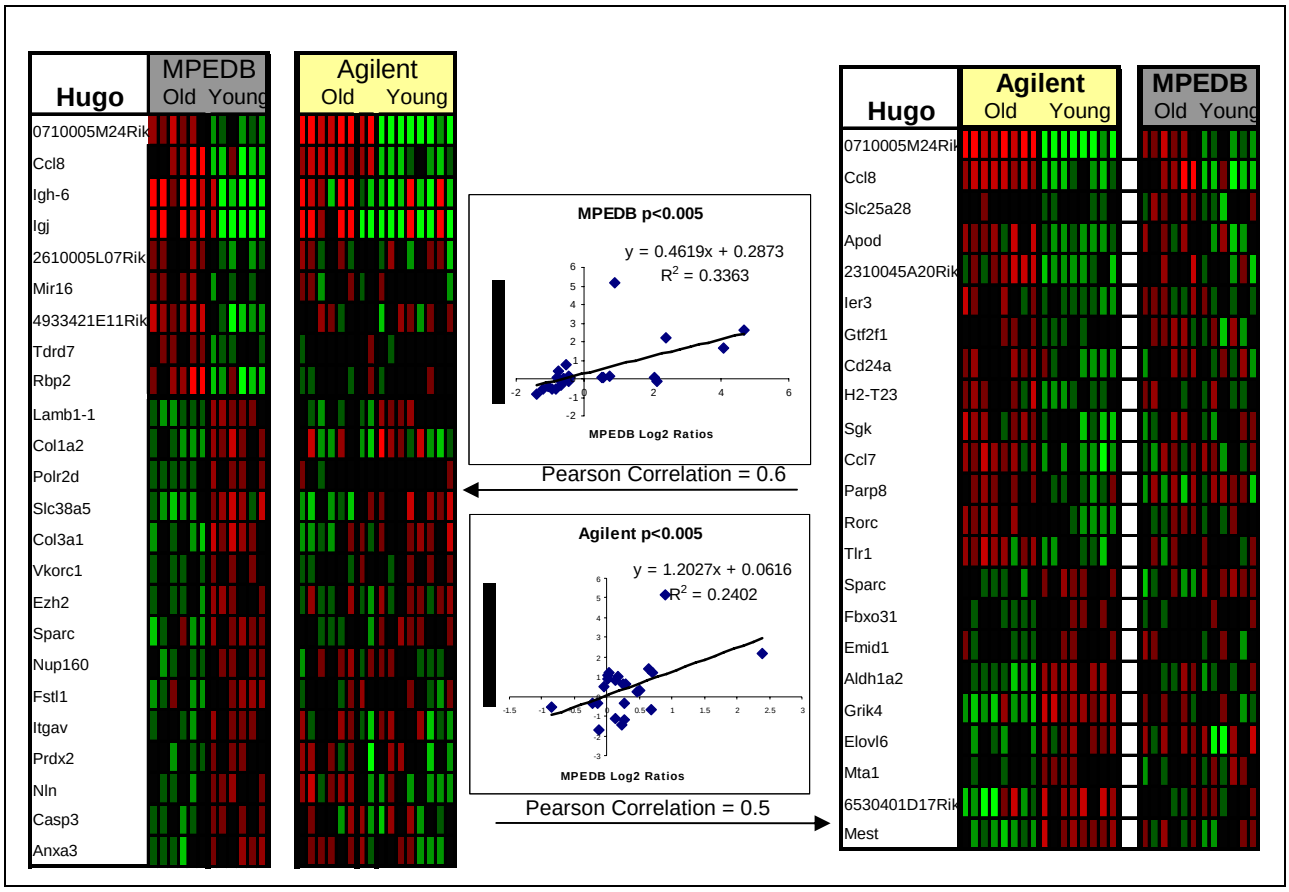


**Figure 6.** Extracellular matrix alterations. A) qRT-PCR for Col1a2 and Col3a1 using RNA extracted from prostate tissue from 4- 12- and 24-months old mice. Ribosomal protein S16 expression levels were used to normalize qRT-PCR data. Normalized results are expressed relative to the lowest expressing value. B) Collagen Type I immunofluorescent staining of frozen sections from anterior prostate lobes from 3 mice at 4- and 24-months of age (Magnification: x40). Note the coarse appearance and less regular distribution of collagen fibers in old prostates compared to the fine collagen fibers and highly organized network in the young prostate. C) Qualitative and Quantitative analysis for the appearance of collagen fibers from young (n=3; 4 images per mouse) and old (n=3; 4 images per mouse) from Collagen Type I immunofluorescent images. ** P-values <0.0001; * P-values <0.005.

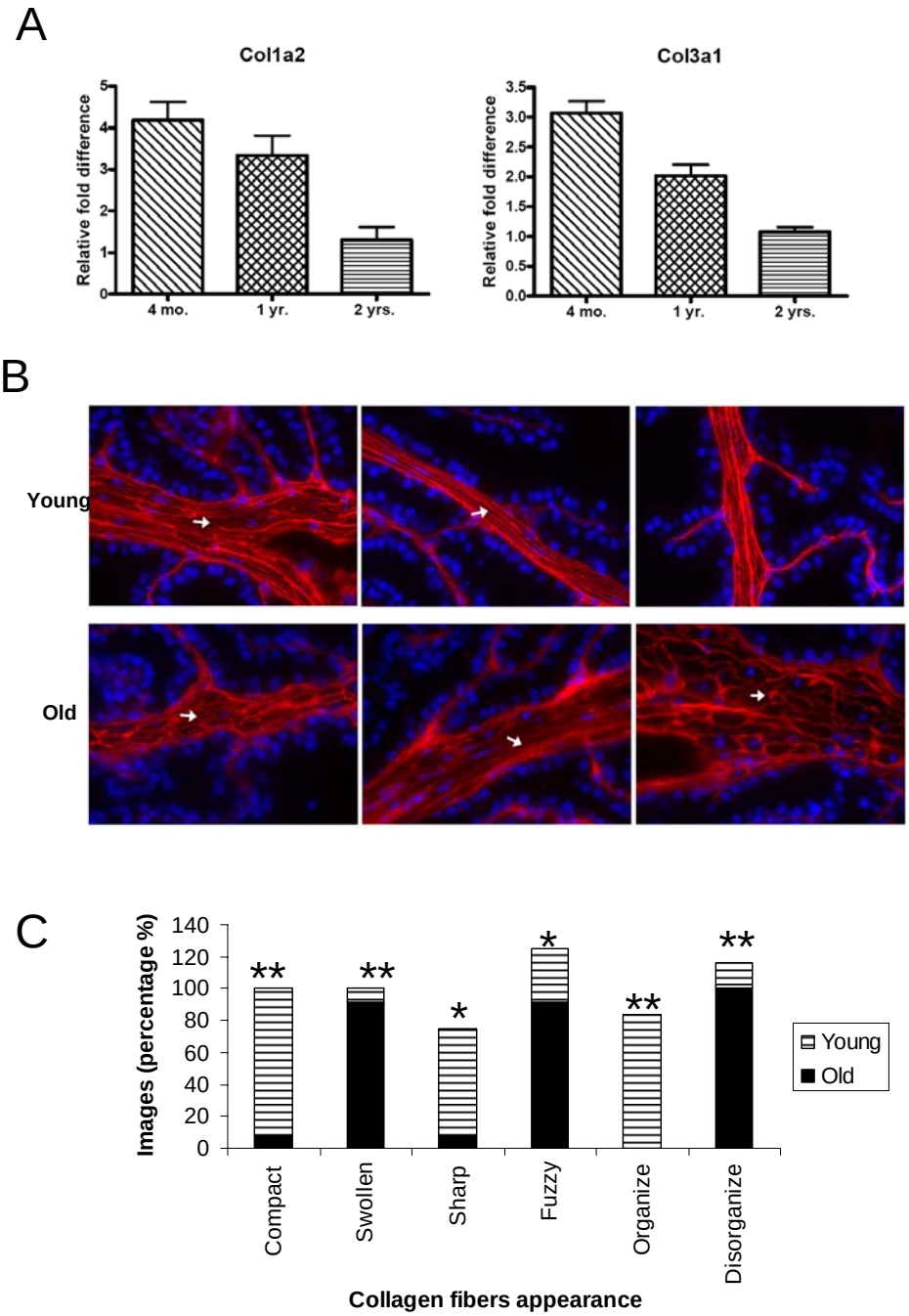


**Figure 7**. qRT-PCR analysis for mouse and human APOD. A) measurement of ApoD transcript levels from RNA isolated from mouse LCM stroma from anterior and dorsal lobes from young (n=8 4-months of age) and old (n=8; 24-months of age) mice. Ribosomal protein S16 expression levels were used to normalize qRT-PCR data. B) measurement of APOD transcript levels from RNA isolated from human pre- and senescent prostate fibroblast. Pre-SEN: pre-senescent cells; SEN(ASH) cell induced to senesce by H2O2; SEN(p16) cell induced to senesce by overexpressing p16. YWHAZ expression levels were used to normalize qRT-PCR data.
Normalized results are expressed relative to the lowest expressing value

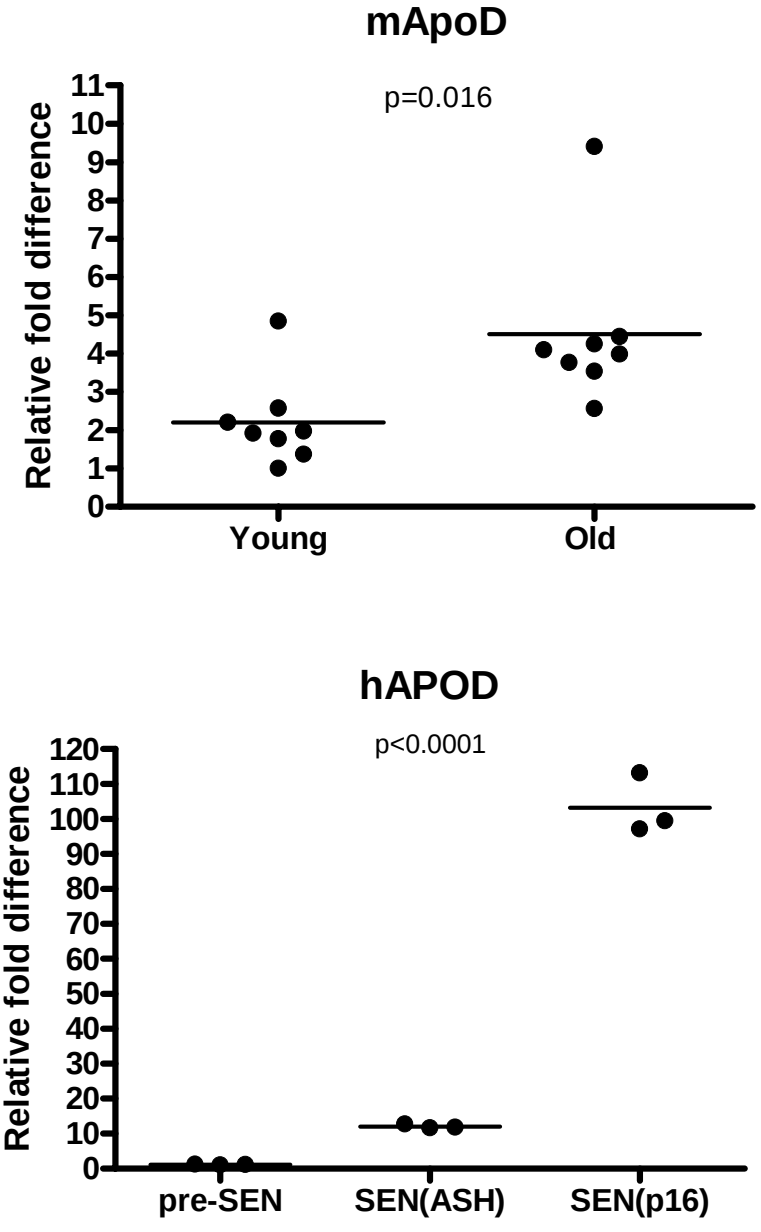


**Figure 8.** Immunohistochemical analysis of 4uM paraffin sections from ventral prostate of 24-months-old mice. Sections were stained with anti-CD45,anti-CD3 and anti B220 anti-F4/80, which recognize general leukocytes, T-cell, B-cells and macrophages respectively. IHC demonstrated a high number of inflammatory cells within the aged prostate tissue.